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Evaluation of Diagnostic Applications of Monoclonal Antibodies against Avian Influenza H7 Viruses▽

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A panel of monoclonal antibodies (MAbs) was generated from mice immunized with binary ethylenimine (BEI)-inactivated H7N1 (A/TK/ON/18-2/00) virus. Using a dot blot assay, six of seven MAbs reacted with viruses of the H7 subtype, but not with any of the other 15 hemagglutinin (HA) subtypes tested. Four of the seven MAbs reacted with 14 different H7 isolates, indicating that the MAbs binding epitopes are conserved among viruses of the H7 subtype. The binding epitopes of all seven MAbs were conformational and reacted with the HA1 fraction of the HA protein in Western blots under nonreducing conditions. Applications of these MAbs in the development of rapid tests for H7 subtype viruses were evaluated. The MAbs demonstrated reactivity with AI virus H7 antigen in immunofluorescence and immunohistochemistry assays. Monoclonal antibody 3 showed a very strong immunostaining in the formalin-fixed and paraffin-embedded tissue from the H7N3 virus-infected chicken. A double-antibody sandwich (DAS) enzyme-linked immunosorbent assay (ELISA) was developed using two of the MAbs. The DAS ELISA specifically detected all H7 strains tested in this study. A competitive ELISA (cELISA) for the detection of H7-specific antibodies was evaluated using one MAb and BEI-inactivated H7N1 virus as the antigen. All infected birds showed positive antibody responses at 7 days postinfection. The sensitivity of this cELISA was comparable with that of an influenza A nucleoprotein-based cELISA. This panel of MAbs is valuable in the development of various immunoassays.

Influenza viruses are a member of the *Orthomyxoviridae* family. The viruses are classified into types A, B, and C on the basis of the antigenic characteristics of the nucleoprotein (NP) and matrix protein (MP) (11, 20). Avian influenza (AI) viruses belong to type A and are further classified into 16 hemagglutinin (HA) and 9 neuraminidase (NA) subtypes based on two surface glycoproteins, HA and NA (7). Influenza A viruses are further classified into low-pathogenic (LPAI) and high-pathogenic AI (HPAI) viruses based on their pathogenic properties in chickens. Infections with viruses of the H5 and H7 subtypes are of the most concern, because they are associated with highly pathogenic outbreaks in poultry. LPAI viruses of the H5 and H7 subtypes may become highly pathogenic after introduction into domestic poultry, as a result of an insertion of basic amino acids into the hemagglutinin cleavage site that facilitates systemic virus replication (29, 31). Lee et al. (13), using helper virus-based reverse genetics, identified specific HA cleavage site sequences that were preferentially incorporated into the low-pathogenic H7 viruses.

AI viruses of the H7 subtype have been classified into three geographically distinct genetic lineages, North American, Eurasian, and Australian (1). H7 viruses from all three lineages have been responsible for a number of HPAI virus outbreaks in domestic poultry in Europe, Asia, North and South America, and Australia. In 1997–1998, an outbreak of subtype H7N2 in Pennsylvania led to the infection of 2.6 million birds (36). Between 1999 and 2000, several outbreaks of avian influenza caused by HPAI H7N1 and LPAI H7N3 viruses occurred in poultry in regions of Northern Italy (4). Infection of humans with H7 subtypes of AI virus has been described (17). An outbreak of highly pathogenic AI H7N7 virus in poultry farms in the Netherlands in 2003 caused 89 human infections and 1 death (3, 12). In 2002 and 2004, outbreaks of HPAI virus due to H7N3 in domestic poultry were reported in Chile and Canada, respectively (31, 33). Human infections with H7N3 virus occurred during the Canadian outbreak (33). A serological analysis of serum samples collected from individuals exposed to AI viruses of the H7 subtype suggested bird-to-human transmissions (24).

The emergence of potentially pandemic H7 strains is possible. Specific surveillance systems are required not only during outbreaks caused by HPAI viruses but also when LPAI viruses are circulating. Therefore, the development of accurate, rapid, and simple diagnostic techniques for AI diagnosis is important. Effective diagnostic tools are also needed to differentially diagnose AI virus infections with the different HA and NA subtypes in the field.

The reliability of immunoassays for viral detection depends on the quality of the immune reagents used. Inconsistencies in the availability and quality of viral antisera have been a major barrier to the development of viral diagnostic capabilities in laboratories. To develop rapid and specific diagnostic methodologies, seven monoclonal antibodies (MAbs) against AI H7 subtype virus were produced and evaluated for their applications in various immunoassays.

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MATERIALS AND METHODS

Preparation of viruses. All viral manipulations were performed under appropriate biosafety level 3 laboratory conditions. Specific-pathogen-free embryonated chicken eggs (9 days old) were inoculated via the allantoic cavity with 0.2 ml of A/TK/ON/18-2/00 virus. Eggs were incubated at 37°C and 55% relative humidity and were monitored twice daily for embryo mortality. Embryos which died within 24 h were discarded. Allantoic fluid from embryos that died after 24 h was collected aseptically and tested by a hemagglutination test.

For mouse inoculation, allantoic fluid containing the H7N1 (A/TK/ON/18-2/00) virus antigen was pooled and clarified by centrifugation at 5,000 rpm (4,600 × *g*), using a JLA 10.5 rotor for 30 min at 4°C. The clarified supernatant was collected, and the virus was inactivated using binary ethylenimine (BEI) to a final concentration of 0.01 M for 24 h at 37°C. The allantoic fluid was filtered through a 0.8-μm filter. The virus was pelleted by centrifugation at 40,000 rpm (160,000 × *g*) in a Ti70 rotor for 50 min and resuspended in NTE buffer (0.1 M NaCl, 10 mM Tris-HCl, 1 mM EDTA [pH 7.5]).

Production of MAbs. The procedures for mouse immunization and monoclonal antibody (MAb) production were performed as previously described (40). Briefly, female BALB/c mice were inoculated subcutaneously with 20 μg of BEI-inactivated and concentrated H7N1 virus (A/TK/ON/18-2/00) in an equal volume of TiterMax Gold (TiterMax USA, Inc., Norcross, GA). Two to three identical boosts were given at 4-week intervals. Mice were boosted with the same antigen in phosphate-buffered saline (PBS) by intravenous injection 3 to 4 days before fusion. Immunized spleen cells were fused with myeloma cells (P3X63 Ag8.653). After 2 weeks, hybridoma supernatants were screened in an indirect enzyme-linked immunosorbent assay (ELISA) using H7N1 as the antigen. The positive clones were subcloned using a limiting dilution technique. Isotyping was performed using a mouse monoclonal antibody isotyping kit (Roche, Indianapolis, IN). All mouse inoculation procedures were performed based on the Canadian Science Centre for Human and Animal Health standard operating procedures under ISO 17025.

Dot blot assay. Viruses representing 16 H subtypes and 14 H7 strains and the negative controls were blotted onto nitrocellulose (NC) membranes (Bio-Rad, San Francisco). After being blocked with 5% skim milk in PBS, the membranes were incubated with 1:5-diluted hybridoma culture supernatants in blocking buffer (with 0.05% Tween 20), followed by incubation with horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (Southern Biotech, Birmingham, AL). Antibody binding was detected using 3,3′-diaminobenzidine (DAB; Sigma-Aldrich, St. Louis, MO). The membrane was washed three times for 10 min with the washing buffer (0.05% Tween 20 in PBS) between each step.

Western blot assay. Electrophoresis was performed using Novex Pre-Cast gels (Invitrogen). Purified H7N1 virus (A/TK/ON/18-2/00) was mixed with lithium dodecyl sulfate (LDS) sample buffer (Invitrogen) with or without dithiothreitol (DTT). Viral proteins were separated by 4 to 12% Bis-Tris gels using a Mini-Protein II apparatus (Bio-Rad) and transferred onto NC membranes. The membranes were blocked with a blocking buffer and then incubated with either purified MAbs or a polyclonal serum in blocking buffer overnight at 4°C. Antibody binding was detected by incubation with HRP-conjugated goat anti-mouse IgG or HRP-conjugated anti-chicken IgG (Southern Biotech) and then the substrate diaminobenzidine (DAB).

HI assay. All MAbs were tested for hemagglutination inhibition (HI) activity using the standard microtiter plate HI assay according to the World Organisation for Animal Health (OIE) manual (37). The HI assay was performed using 4HA units of H7N1 A/TK/ON/18-2/00 virus and chicken erythrocytes.

Virus neutralization test. Two hundred 50% egg-infectious doses (EID_{50}) of H7N3 A/CK/BC/CN00007/04 was combined with equal volumes of 2-fold serially diluted hybridoma culture supernatants and incubated for 60 min at 37°C. These were then used to inoculate 9-day-old chicken embryos via the allantoic cavity. Embryo mortality was monitored twice daily. Allantoic fluids from dead and live embryos at the end of the experiment were harvested and tested for HA activity. Virus neutralization titers were determined according to the OIE manual (37).

Immunohistochemistry and immunofluorescence assays. The procedures for immunohistochemistry and immunofluorescence assays were performed as previously described (39). Briefly, Madin-Darby canine kidney (MDCK) cells were grown on microscope slides. The cells were infected with HPAI H7N3 virus A/CK/BC/CN00007/04 (other strains were not examined) at a multiplicity of infection (MOI) of 0.1. After 18 h, cell monolayers were fixed by immersing the slides in Coplin jars filled with paraformaldehyde solution (3.7% formaldehyde and 0.2% Triton X-100 in PBS) and incubated at 37°C for 30 min. Cells were then blocked with 10% goat serum in PBS for 1 h at 37°C before being incubated separately with each of the seven hybridoma culture supernatants for 1 h at room temperature in a humidified chamber. For immunofluorescence, cells were incubated with goat anti-mouse IgG conjugated with Alexa Fluor 594 (Molecular Probes, Eugene, OR). The microscope slides were mounted with an anti-fade reagent in glycerol buffer (Molecular Probes, Eugene, OR), and fluorescence was visualized with an Olympus FluoView laser scanning confocal microscope (Olympus, Melville, NY).

For the immunohistochemistry assay, an archived tissue sample collected during the HPAI H7N3 virus outbreak in Saskatchewan, Canada (H7N3 A/CK/Saskatchewan/HR-00011/07), was used (2). The paraffin-embedded tissue was sectioned (5 μm), mounted on microscopic slides, and quenched for 10 min in aqueous 3% H_2O_2 and then pretreated with proteinase K for 15 min. Slides containing the MDCK cells and cerebral tissue were incubated with purified MAbs overnight at 4°C, visualized using an HRP-labeled polymer, EnVision+ system (anti-mouse) (Dako), and reacted with the chromogen DAB. The sections were then counterstained with Gill's hematoxylin.

Purification and biotinylation of MAbs. The procedures for MAb purification and biotinylation were performed as previously described (39). Briefly, the subcloned hybridomas were grown for 7 days in roller bottles. The supernatants were then harvested and concentrated. The MAbs were purified from hybridoma culture supernatants using a HiTrap Protein G affinity column (Amersham Biosciences, Uppsala, Sweden).

The purified MAbs were concentrated and dialyzed against 0.1 M $NaHCO_3$, pH 8.4, at 4°C. A biotin spacer conjugate (D-biotinoyl-ε-aminocaproic acid *N*-hydroxysuccinimide ester; Roche, Indianapolis, IN) was dissolved in anhydrous dimethyl sulfoxide (DMSO; Sigma-Aldrich) at 8 mg/ml and added to the antibody. The reaction was allowed to proceed for 4 h at room temperature in the dark. The unbound biotin was then removed by extensive dialysis against PBS at 4°C.

Double-antibody sandwich (DAS) ELISA for H7 antigen detection. Microtiter plates were coated with purified MAb 3 (F28-31, 1.6 μg/ml) diluted in a 0.06 M carbonate/bicarbonate buffer, pH 9.6, and incubated overnight at 4°C. The plates were blocked with 5% skim milk in PBS with 0.05% Tween 20 at 37°C for 1 h. The AI viral antigens were added to the plates. After incubation, biotin-conjugated MAb 7 (F39-16, 0.25 μg/ml), followed by HRP-conjugated streptavidin (1:2,000), was added to each well. After an incubation step, *o*-phenylenediamine dihydrochloride (OPD; Sigma-Aldrich) was added. An equal volume of 2.0 M sulfuric acid was used to stop the color reaction. The optical density (OD) was measured at 490 nm using an automated plate reader (Photometer Multiskan reader; Labsystems, VA). Each incubation step was for 60 min at 37°C with gentle shaking, followed by three washes with a washing buffer after each incubation.

Experimental sera. All procedures involving experimental animal inoculations and care complied with the Canadian Council of Animal Care guidelines. The serum samples used in competitive ELISA (cELISA) were obtained from turkeys infected with 10^6 EID_{50} of different influenza viral subtypes. An additional six turkeys were inoculated with 10^6 EID_{50} of H7N1 A/TK/ON/10-2/00, and the sera were collected at different days postinfection (dpi).

cELISA for H7-specific antibody detection. A cELISA was performed as previously described (38). Briefly, microtiter plates (Nunc-Immunoplate, Roskilde, Denmark) were coated with BEI-inactivated H7 virus (50 μl/well) in carbonate buffer (pH 9.6) overnight at 4°C. After the washing step, equal volumes of diluted test sera (1:5) and a hybridoma culture supernatant (designated no. 7, F39-16, 1:1,600) were added to the plates, which were incubated at 37°C for 1 h with agitation. HRP-conjugated anti-mouse IgG was then added, and the mixture was incubated for 1 h at 37 °C, followed by a washing step. The OPD was added, and color development was stopped after 15 min by adding 50 μl of 2.0 M sulfuric acid per well. The OD was determined at 490 nm by using an automated plate reader.

Results were expressed as the percentage of inhibition (PI), derived by the following formula: PI = [(negative reference serum OD – test sample OD)/(negative reference serum OD – positive reference serum OD)] × 100%. A cutoff value of 50% inhibition was established based on testing of known negative sera.

RESULTS

Monoclonal antibodies. The fusions were performed using mouse spleen cells inoculated with BEI-inactivated H7N1 virus (A/TK/ON/18-2/00). Three fusions allowed the production of seven hybridomas against H7 viral antigen. After the subcloning, the MAbs were designated and their isotypes were characterized (Table 1). Dot blot assays were used to examine the

TABLE 1. Characterization of the MAbs produced against AI H7 virus[a]

MAb no.	Clone	Isotype	Result for assay indicated							
			HI	VNT	IHC		IF	Western blot		cELISA
					Cells	Tissues		HA0	HA1	
1	F28AIH7N1-68	IgG2a/κ	+	+	+	+	+	+	+	−
2	F28AIH7N1-80	IgG2a/κ	+	+	+	+	+	+	+	−
3	F28AIH7N1-31	IgG2a/κ	+	+	+	++	+	+	+	+
4	F28AIH7N1-83	IgG1/κ	+	+	+	−	+	+	+	−
5	F39AIH7N1-9	IgG2a/κ	−	−	+	+	+	+	+	+
6	F39AIH7N1-13	IgG1/κ	−	+	+	+	+	+	+	+
7	F39AIH7N1-16	IgG2b/κ	−	+	+	+	+	+	+	+

[a] VNT, virus neutralization test; IHC, immunohistochemistry; IF, immunofluorescence; −, negative; +, positive, ++, strongly positive.

specificity of the MAbs against different AI virus subtypes and diverse H7 isolates. Sixteen subtypes (H1 to H16) and 14 H7 strains were assayed using the seven MAbs. The results indicate that six of the seven MAbs reacted only with H7 (Table 2), the exception being MAb 6, which also reacted with H10N8 A/Quail/ITHACA/1117/65. However, further testing showed that MAb 6 did not react with H10N7 A/CK/GERMANY/N/49, suggesting that MAb 6 recognized a shared epitope with some but not all viruses of the H10 subtype. Four MAbs (3, 5, 6, and 7) reacted with all 14 H7 strains tested (Table 2). These data demonstrate that the binding epitopes of the four MAbs are conserved among H7 strains. Monoclonal antibodies 1, 2, and 4 failed to recognize one strain from the European lineage, H7N3 A/TK/ENG/63.

TABLE 2. Summary of the MAb reactivities with different H7 virus strains and subtypes in the dot blot assays

AI virus H7 subtype and isolate[a]	Result for MAb indicated						
	1	2	3	4	5	6	7
(a) H7N3 A/TK/OREGON/71	+	+	+	+	+	+	+
(b) H7N3 A/TK/ENG/63	−	−	+	−	+	+	+
(c) H7N1 A/TK/ON/18-2/00	+	+	+	+	+	+	+
(d) H7N3 A/DK/AB/4012-567/06	+	+	+	+	+	+	+
(e) H7N3 A/DK/BC/3578/07	+	+	+	+	+	+	+
(f) H7N3 A/DK/AB/4012-532/06	+	+	+	+	+	+	+
(g) H7N2 A/TK/ORE/PORTCHAL/73	+	+	+	+	+	+	+
(h) H7N3 A/CK/BC/CN00007/04	+	+	+	+	+	+	+
(i) H7N3 A/CK/BC/1827/07	+	+	+	+	+	+	+
(j) H7N3 A/CK/AUST/3634/92	+	+	+	+	+	+	+
(k) H7N3 A/Teal/GERMANY/07	+	+	+	+	+	+	+
(l) H7N3 A/Swan/POTSDAM/07	+	+	+	+	+	+	+
(m) H7N7 A/Mallard/GERMANY/07	+	+	+	+	+	+	+
(n) H7N1 A/Mallard/NVP/41/61	+	+	+	+	+	+	+
H5N9 A/TK/WIS/68	−	−	−	−	−	−	−
H5N3 A/TK/CA/35621/84	−	−	−	−	−	−	−
H1N1 A/Swine/IOWA/31/97	−	−	−	−	−	−	−
H2N3 A/Mallard/Al/77/77	−	−	−	−	−	−	−
H3N8 A/Parakeet/NETH/22-3/00	−	−	−	−	−	−	−
H4N6 A/DK/CZECH/56	−	−	−	−	−	−	−
H6N8 A/TK/ON/63	−	−	−	−	−	−	−
H7N3 A/CK/BC/514/04	+	+	+	+	+	+	+
H8N4 A/TK/MN/128/77	−	−	−	−	−	−	−
H9N2 A/TK/MN/12877/1285/81	−	−	−	−	−	−	−
H10N8 A/Quail/ITHACA/1117/65	−	−	−	−	−	+	−
H11N9 A/Tern/AUSTRALIA/G70C/75	−	−	−	−	−	−	−
H12N5 A/DK/AB/1607/76	−	−	−	−	−	−	−
H13N6 A/Gull/MD/704/77	−	−	−	−	−	−	−
H14N5 A/Mallard/ASTRAKHAN/263/82	−	−	−	−	−	−	−
H15N8 A/DK/AUSTRALIA/341/83	−	−	−	−	−	−	−
H16N3 A/Black-headed gull/SWEDEN/5/99	−	−	−	−	−	−	−

[a] Isolates labeled a through n are referred to as such in the text.

Characterization of MAb binding sites. Seven H7 MAbs were tested for virus neutralization and HI activities. Six MAbs demonstrated neutralizing activities against H7N3 (A/CK/BC/CN00007/04), and MAbs 1, 2, 3, and 4 showed HI activity against homologous virus H7N1 (A/TK/ON/00). MAb 5 did not demonstrate any HI or neutralization activities. The positive neutralizing and HI activities of these MAbs indicated that they are HA specific.

In order to further confirm the specificity, the reactivity of the MAbs to H7N1 virus was analyzed by Western blot assays. The H7N1 (A/TK/ON/10-2/00) viral protein was separated using Bis-Tris gels and transferred to NC membranes. The specific reactions were detected using the MAbs and an H7-specific polyclonal antiserum as a positive control. None of the H7 MAbs reacted with denatured viral antigen (data not shown), suggesting that they may recognize only conformational epitopes. Although SDS-PAGE/Western blot analysis is a valuable approach for characterizing MAb binding sites, denaturing conditions are not suitable for conformational epitope identification. Under nonheated conditions with LDS in the sample buffer, all seven MAbs and the polyclonal chicken antiserum recognized an approximately 65-kDa protein band, which corresponds to HA0. Only the polyclonal antiserum and MAb 5 are shown in Fig. 1. The high-molecular-weight bands recognized by the MAbs and the polyclonal antibody likely correspond to the stable HA trimer (Fig. 1, lanes 2 and 4). With the addition of DTT to the sample buffer, all MAbs and the polyclonal antiserum reacted with HA0 and a protein band with a molecular mass of approximately 42 kDa, corresponding to HA1. The key factor in maintaining the reactivity of the epitope appears to be the elimination of boiling prior to electrophoresis. The presence of LDS and DTT in the sample buffer without heating did not affect reactivity of the epitope. In conclusion, the results from the Western blot analyses indicate that all MAbs are HA1 specific.

Immunohistochemistry and immunofluorescence assays for detecting H7 antigen. Immunohistochemistry and immunofluorescence assays were performed to examine whether the MAbs can identify H7 viral antigen in infected cells. Results from both immunohistochemistry and immunofluorescence assays indicate that all seven MAbs were able to specifically recognize the viral antigen with low background in H7N3

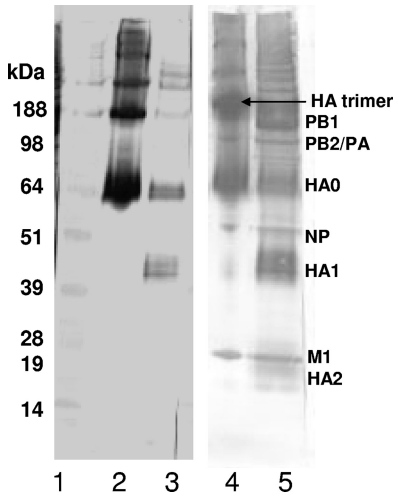

FIG. 1. Western blot analysis using MAb 5 and a polyclonal serum. H7N1 (A/TK/ON/10-2/00) viral proteins were separated by a 4 to 12% Bis-Tris gel. The protein was transferred to an NC membrane and assayed using MAb 5 (lanes 2 and 3) and a polyclonal serum from a chicken infected with H7N1 virus (A/TK/ON/10-2/00) (lanes 4 and 5). Lane 1 shows molecular mass standards (10^3 kDa). Lanes 2 and 4 are H7N1 viral proteins in LDS sample buffer. Lanes 3 and 5 are H7N1 viral proteins in LDS sample buffer with DTT. PB1, PB2, and PA, heterotrimer polymerase complex; M1, matrix protein.

A/CK/BC/CN00007/04 virus-infected MDCK cells (Fig. 2a and b).

To test if the MAbs can detect H7 antigen in formalin-fixed, paraffin-embedded tissues, archived cerebrum collected from a chicken during the 2007 HPAI H7N3 outbreak in Saskatchewan, Canada, was used (2). Six of seven MAbs were able to detect the H7 antigen, while none of the MAbs showed cross-reactivity with negative-control cerebral tissue. MAb 4 (F28-83) failed to react with the H7 antigen. In contrast to the anti-NP MAb (38), which showed mostly intense nuclear staining with lesser diffuse cytoplasmic staining (Fig. 3a), the H7 MAbs reacted with influenza virus antigen that was distributed diffusely throughout the cytoplasm and processes of neurons (Fig. 3b). The MAb 3 (F28-31) showed a particularly strong reactivity with viral antigens (Fig. 3b), which is valuable for routine immunohistochemical diagnosis.

DAS ELISA for H7 viral antigen detection. The MAbs were evaluated for their ability to detect H7-specific viral antigen in the development of a DAS ELISA. Since MAbs 3, 5, 6, and 7 reacted with all 14 of the tested H7 strains, they were examined for the best pair combination by using dot blot assays. The four purified MAbs were blotted onto four separate NC membranes and incubated with H7 viral antigen. The four biotinylated MAbs were then used to detect antigen binding. The antibody

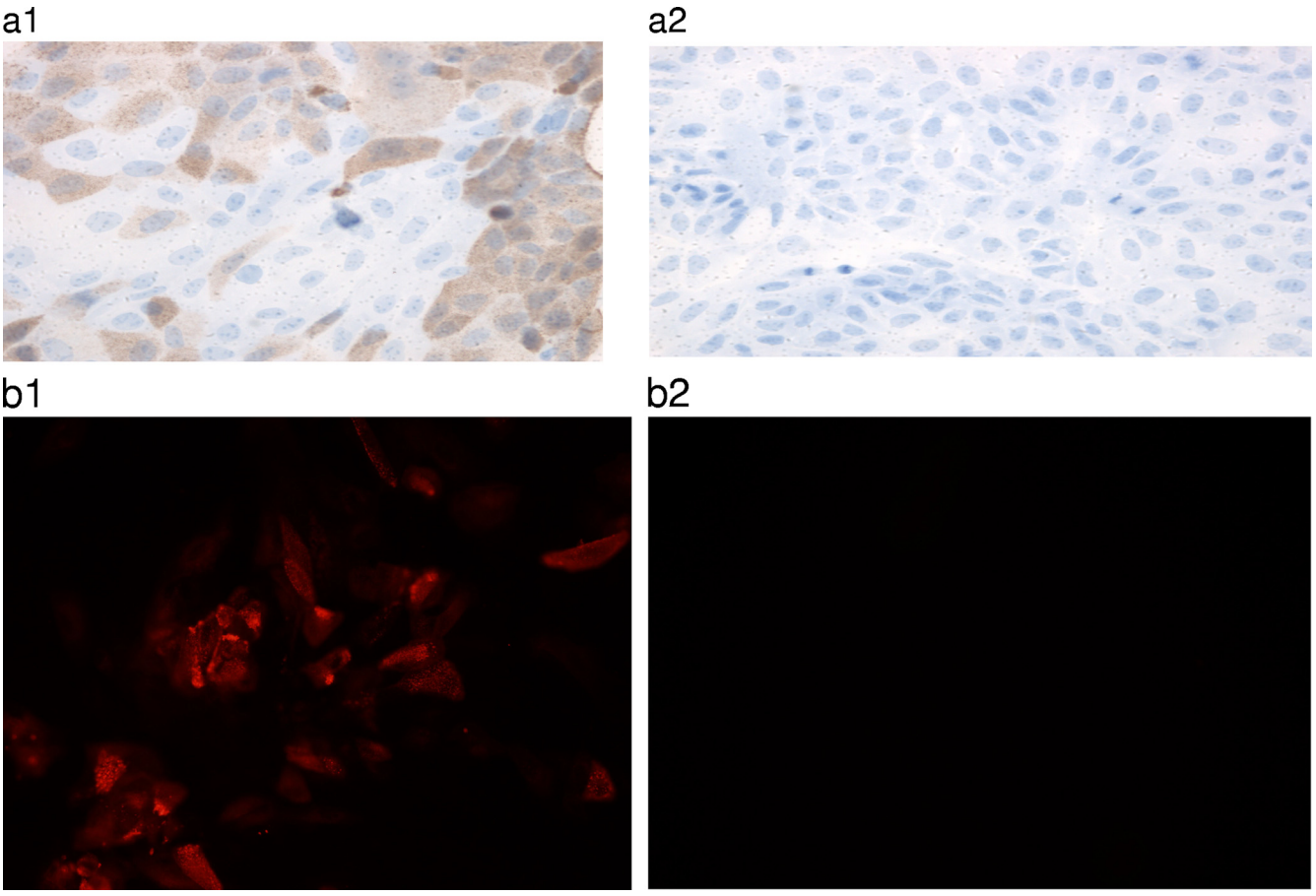

FIG. 2. Immunohistochemistry (a) and immunofluorescence (b) analyses of AI virus in infected cells using MAbs. MDCK cells were infected with H7N3 (A/CK/BC/CN00007/04) virus. The cells were fixed in formalin and blocked. The viruses were assayed with antibodies. a1 and b1 were detected with MAb 3, and a2 and b2 were negative controls (without the MAb).

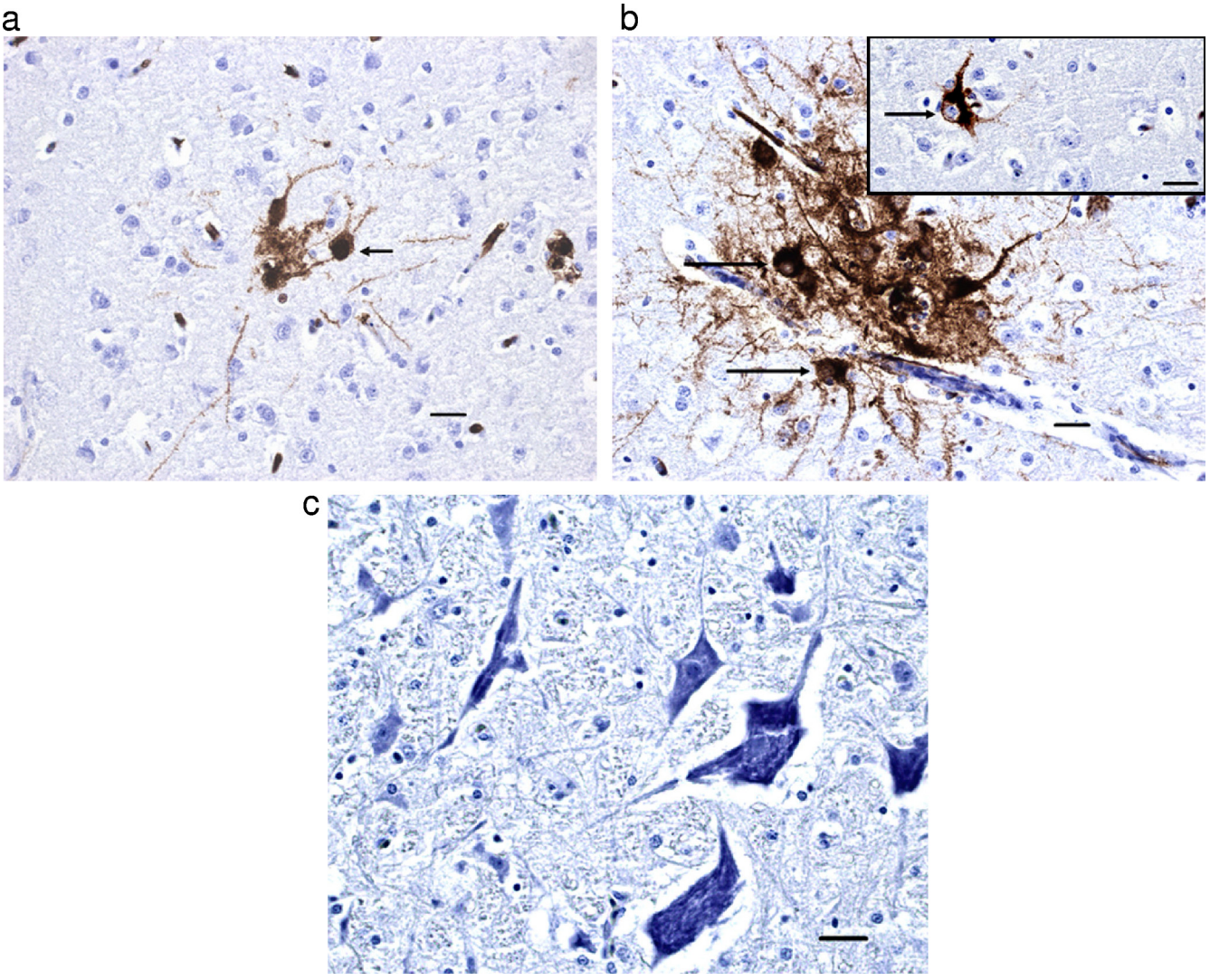

FIG. 3. Immunohistochemistry analysis of H7 subtype virus-infected cerebral tissue. Archived, paraffin-embedded cerebral tissue collected during the HPAI H7N3 virus outbreak in Saskatchewan (H7N3 A/CK/Saskatchewan/HR-00011/07) was used. The viral antigens were assayed with MAbs. (a) Anti-NP-specific MAb (arrow, intense nuclear staining). (b) MAb 3 (F28-31) (arrow, strong reactivity in cytoplasm and axons of neurons). Inset, individual neuron with specific cytoplasmic staining and unstained nucleus. (c) Negative control (cerebral tissue). Bars = 20 μm.

pair made up of MAbs 3 and 7 showed the strongest signal compared with that of the others (data not shown). Therefore, MAb 3 was used as the capture antibody and MAb 7 was used as the detector antibody in the DAS ELISA.

DAS ELISA demonstrated a positive reaction only with H7 antigen and not with other HA subtypes (Fig. 4a), thus implying that it is specific for the H7 subtype. The analytic sensitivity of the test was determined to be equivalent to HA titers of ≥4 (data not shown). The ability of the DAS ELISA to detect different H7 strains was also examined. The results indicated that the DAS ELISA was able to identify all 13 AI H7 virus strains tested (Fig. 4b) (note that the H7N1 A/DK/AB/4012-567/06 strain listed in Table 2 [isolate d] was not tested).

Detection of H7-specific antibodies using cELISA. With the intention of developing a sensitive cELISA for H7 antibody detection, the seven MAbs were examined for their ability to compete with polyclonal antisera collected from birds that were experimentally infected with viruses of the H7 subtype. Four of the seven MAbs demonstrated the ability to compete with positive polyclonal antiserum from a bird infected with the H7 subtype virus (Table 1). Monoclonal antibody 7 was subsequently selected as a competitor MAb for the cELISA. The H7 antigen concentrations and the MAb dilutions were then optimized to obtain a large range in OD values for positive and negative sera.

To determine the analytic specificity of the cELISA, sera from birds infected with HA subtypes H1 to H16 (Table 3) were evaluated using the cELISA. The results indicated that binding of MAb 7 to H7 antigen was inhibited only by the anti-H7 antiserum and not by the antisera against other HA subtypes (Fig. 5a). These results confirmed that the cELISA was specific for detecting antibodies to the H7 subtype. Since

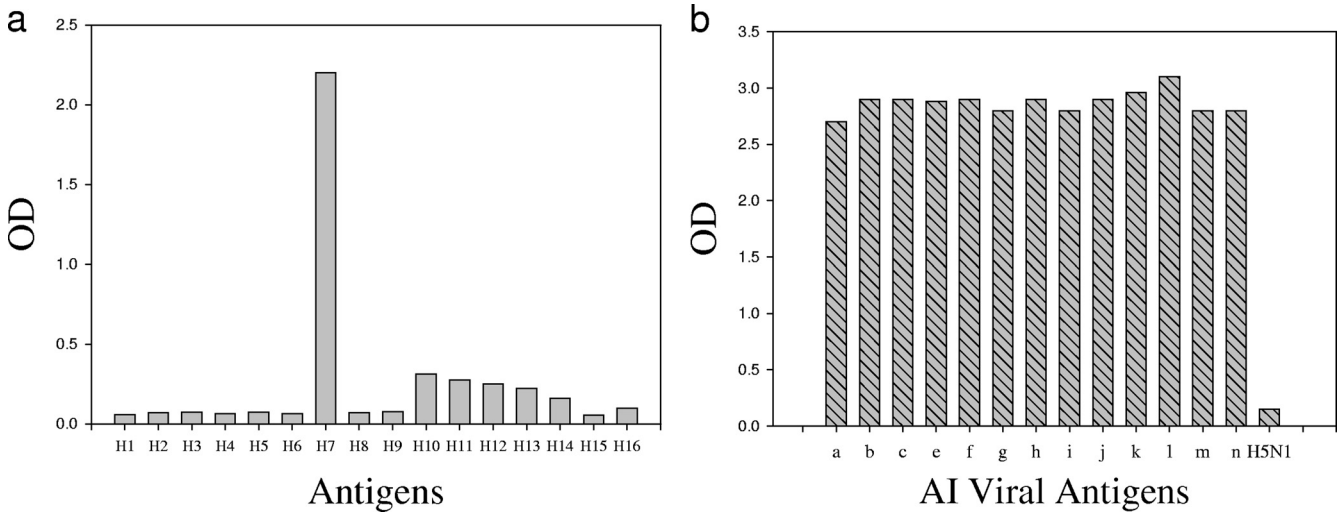

FIG. 4. Detection of different subtypes and H7 strains using the DAS ELISA. Purified MAb 3 (F28-31) was used to coat 96-well plates. (a) H1-H16 AI viral antigens (Table 2). (b) The 13 H7 viral isolates (a to n in Table 2; isolate d was not tested) and a negative control (H5N1) were added to the plates, and the viral antigens were detected with biotin-conjugated MAb 7.

MAb 7 reacted with all 14 H7 strains tested (Table 2), it is hypothesized that MAb 7 may have a broad specificity with a large number of H7 antisera.

Sera obtained from experimentally infected turkeys were tested by cELISA. All infected birds showed positive H7-specific antibody responses (>50% inhibition) as early as 7 dpi, and sera continued to test positive until the end of the experiment on day 28 (Fig. 5b). The cELISA results showed a high concordance with the test results using an NP-based cELISA (41) and an agar gel immunodiffusion assay (AGID) (37) (Table 4). This indicates that the sensitivity of the cELISA is comparable to that of these tests. It can be concluded that the cELISA is H7 subtype specific and can be used for detecting H7-specific antibodies in infected sera (the validation, standardization, and application of this cELISA will be published in a separate paper).

TABLE 3. Sera collected from birds infected with different AI virus subtypes

Virus used for infection	Collection time (dpi)
H1N1 A/CK/BC/3/98	22
H2N9 A/Pintail/AB/293/77	26
H3N2 A/DK/ON/05/00	7
H4N6 A/DK/BC/14/99	7
H5N2 A/DK/BC/26-6/05	26
H6N1 A/TK/ON/849-2/06	18
H7N3 A/CK/BC/CN00007/04	13
H9N2 A/TK/MN/12877/1285/81	21
H10N7 A/CK/GERMANY/N/49	15
H11N1 A/DK/ENG/56	18
H12N5 A/DK/AB/1607/76	14
H13N1 A/Gull/MD/704/77	9
H14N5 A/Mallard/GURJEV/263/82	52
H15N8 A/DK/AUSTRALIA/341/83	10
H16N3 A/Black-headed gull/6/SWEDEN/5/99	35

DISCUSSION

Since the avian influenza H7 subtype is one of two HA subtypes capable of becoming HPAI virus after transmission to domestic poultry, the rapid and early detection of this subtype is essential to controlling outbreaks. In this study, seven MAbs were produced, and their diagnostic applications were evaluated.

The MAbs were produced from mice inoculated with a BEI-inactivated H7N1 virus (A/TK/ON/18-2/00). Six MAbs were shown to react with the H7 subtype but not the other 15 HA subtypes by the dot blot assays. Monoclonal antibody 6 showed cross-reactivity with H10N8 A/Quail/ITHACA/1117/65, but not with another H10 virus isolate (H10N7 A/CK/GERMANY/N/49). The epitope to which MAb 6 binds could be shared in some H10 viruses. Among all the HA subtypes, H7, H15, and H10 sequences are genetically closely related, explaining why MAb 6 cross-reacted with the H10 subtype.

With the purpose of validating the reactivity of the MAbs against different H7 strains, 14 virus isolates of the H7 subtype were tested in a dot blot assay. Of the seven MAbs produced in this study, four (3, 5, 6, and 7) reacted with all 14 H7 virus strains that were tested, indicating that they recognize a conserved epitope(s). Similar observations were reported for three H1-specific MAbs that demonstrated reactivity with two genetic lineages of H1 viruses (34). Although MAbs against an H7N2 subtype virus were produced and characterized by Wang et al. (36), their reactivity against different H7 isolates was not examined.

Even though the HA1 proteins of influenza A viruses exhibit a relatively high degree of antigenic variability, the percentage homology scores of HA1 for three isolates of Eurasian and North American (H7N3 A/TK/OREGON/71 [AB269693], H7N3 A/TK/ENG/63 [CY015065], and H7N3 A/CK/BC/CN00007/04 [EF470587]) lineages are 83.9% at the amino acid level and 74.5% at the nucleotide level. The existence of antigenic ho-

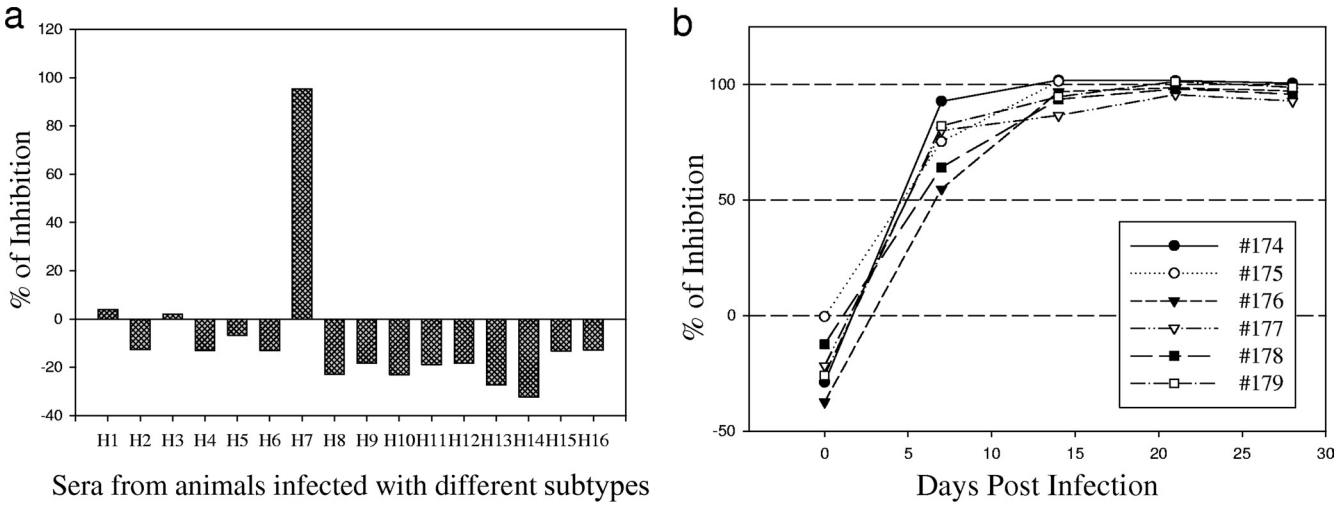

FIG. 5. Detection of the H7-specific antibody response by H7 cELISA. BEI-inactivated H7N1 virus was used to coat microtiter plates. Equal volumes of diluted test sera and MAb 7 (1:1,000) were added to the plates and allowed to compete for the H7 cELISA specificity determination. (a) Sera collected from birds infected with different AI virus subtypes (Table 3). (b) Sera from experimentally infected turkeys. Results are expressed as percentages of inhibition. A cutoff value of 50% inhibition was established based on testing of known negative sera.

mology between Eurasian and North American lineage H7 viruses may explain why four of the MAbs (3, 5, 6, and 7) cross-reacted with all 14 H7 strains that were tested. The other three MAbs (1, 2, and 4) failed to react with European H7N3 A/TK/ENG/63, indicating that this specific European isolate is more antigenically variable than the other 13 H7 strains. The lack of reactivity of three MAbs to this strain may be due to antigenic variations resulting from mutations, short deletions, insertions, RNA reassortment, or changes of glycosylation sites (20).

The advantages of using MAbs for detecting infectious viruses include high specificity and the unlimited supply of a standardized reagent. One potential limitation is that a MAb can react with only a single epitope. However, the binding epitopes for MAbs 3, 5, 6, and 7 appear to be highly conserved among different H7 strains. If MAbs are against highly conserved regions, the risk of false-negative results is significantly reduced.

Four of seven MAbs demonstrated HI activity against the homologous H7N1 virus, and six MAbs possess virus-neutralizing activity. Two MAbs (6 and 7) were able to neutralize virus but did not have HI activity. Similar results have been reported by others (10, 18, 26). The possible explanations are that (i) there are different receptor binding sites for erythrocytes and host cells, (ii) neutralizing MAbs without HI activity bind to the stalk region of the HA and inhibit the fusion activity of HA (18), and (iii) non-HI-neutralizing MAbs inhibit the fusion of virus with the intracellular vacuolar membrane by interfering with the conformational rearrangements (9, 5). Monoclonal antibody 5 gave positive results by ELISA and the dot blot assay and also specifically bound to the HA1 fragment of HA in Western blot analysis but was negative in HI and virus neutralization assays. It is possible that the binding site for this MAb is located on HA1 but is not associated with the ligand binding site on host target cells or sialic acid receptors present on the surface of red blood cells.

Western blot results demonstrate that all seven MAbs

TABLE 4. Comparison of H7 cELISA with AGID and AI-NP cELISA results

H7N1 A/TY/ON/10-2/00 virus-infected serum sample no.	dpi	Result for:		
		H7 cELISA (% inhibition)	AGID[a]	AI-NP cELISA (% inhibition)
174	0	−29	−	−8
	7	93	+	72
	14	101	+	66
	21	101	+	66
	28	100.5	+	69
175	0	−0.5	−	−11
	7	75	+	60
	14	101	+	72
	21	NA[b]	+	86
	28	97	+	85
176	0	−37	−	−12
	7	54	+	46
	14	97	+	58
	21	98	+	62
	28	97	+	82
177	0	−21	−	−14
	7	80	+	59
	14	86	+	72
	21	95	+	73
	28	93	+	75
178	0	−12	−	−9
	7	64	+	48
	14	94	+	60
	21	98	+	74
	28	96	+	76
179	0	−25	−	−12
	7	82	+	68
	14	95	+	80
	21	101	+	82
	30	98	+	87

[a] AGID, agar gel immunodiffusion assay.
[b] NA, not applicable.

recognize conformational epitopes, because of the negative results under reduced conditions. This corresponds to the report that most neutralizing antibodies bind conformational epitopes, which act as antigenic sites on the HA1 globular head domain (15). All seven MAbs reacted with trimeric and monomeric HA and HA1 in a Western blot analysis under nonreduced conditions. The denaturing agent (LDS) and reducing agent (DTT) without heating cleaved the influenza virus particles into their subunits but did not affect the conformational epitopes. Similar results have been reported by Fortsas et al. (6) for MAbs against human adenoviruses. The molecular weights of HA0 and HA1 determined by Western blot analysis in this study are lower than those in the data published previously by Shaw et al. (28), since they analyzed AI virus proteins under reducing conditions. The reducing agent (DTT) with heating cleaves disulfide bonds between cysteine residues and linearizes proteins, completely explaining why the protein molecular weights are higher than those in the results obtained in this study.

Immunohistochemical and immunofluorescence techniques are commonly used for identification of specific viruses. Both technologies make it possible to visualize the distribution and localization of the virus in infected cells or tissues. Rapid diagnoses of influenza A and B virus infection by immunofluorescence using MAbs have been reported (16, 23). In recent H1N1 outbreaks, the direct fluorescent antigen test was able to effectively rule out novel H1N1 infections (22). All seven MAbs developed in this study reacted with the H7 viral antigen in infected cells with low background levels in both immunohistochemical and immunofluorescence assays. Six of seven MAbs showed a positive staining on formalin-fixed and paraffin-embedded H7-positive tissue but on none of the negative-control tissues. The anti-HA MAbs stained mainly cytoplasm and axons of neurons, in contrast to the anti-NP antibody, which stained predominantly cell nuclei. These data agree with the understanding that viral ribonucleoprotein (RNP) is synthesized in the nucleus (8, 19). MAb 3 in particular showed very strong immunostaining, which is useful for the rapid identification of H7 virus isolates.

DAS ELISA for H5 antigen detection offers an advantage over other antigen detection techniques, such as virus isolation and reverse transcription-PCR (RT-PCR), because it is rapid, simple, and specific. An H7N1 antigen capture ELISA that detected tracheal samples as early as 3 days postinfection was reported by Velumani et al. (35). However, their test used a MAb as the capture antibody and a polyclonal guinea pig serum IgG as the detection antibody. Polyclonal antibodies have disadvantages related to heterogeneity, resulting in unexpected cross-reactivity and difficulty in standardization.

The DAS ELISA developed in this study utilizes two H7-specific MAbs, which increases its specificity. Since MAbs 3 and 7 recognize all 14 of the H7 strains tested, they are ideal for use in the DAS ELISA. In addition, these two antibodies recognize different antigenic epitopes on the H7 molecule, since MAb 3 possesses both HI and virus neutralization activities, whereas MAb 7 does not have HI activity. The prototype DAS ELISA described in this study is able to differentiate H7 AI viruses from other AI virus subtypes and detect various H7 strains, while only an H7N1 virus was examined with the H7 capture ELISA described by Velumani et al. (35). The assay can detect H7 virus with an HA titer of ≥ 4, which is similar to detection by an H5-specific DAS ELISA (39). Immunochromatographic strips for rapid detection of influenza viruses using two specific MAbs have been reported for H5, H7, and H9 virus subtypes (14, 32, 21). The pair of MAbs (3 and 7) reacted with all 14 of the H7 strains and has the potential for use in the development of an immunochromatographic strip test for rapid detection of the H7 antigen.

Competitive ELISAs are widely used for serological detection of antibodies to influenza viruses, mainly due to their sensitivity and simplicity (25, 27, 30). Their significant advantage is their species-nonspecific approach. The cELISA makes it possible to provide general assays for testing sera from different avian species, humans, and other species without changing any of the test reagents. It is a challenge to produce MAbs with the same specificity as host antibodies in infected sera for the development of cELISA. However, four of the seven MAbs produced in this study competed with the polyclonal antibody from an infected turkey. Monoclonal antibody 7 was selected in the cELISA, as it showed competition only with monospecific polyclonal sera (H7) and cross-reacted with all H7 strains tested. With this cELISA, 100% of the sera from H7 subtype virus-infected birds showed positive antibody responses at 7 dpi ($>50\%$ inhibition). The results of the H7 cELISA using sera from turkeys experimentally infected with an H7N1 virus showed a high concordance with AGID and NP-based cELISA (Table 4). Correspondingly, Sala et al. (25) also reported a good correlation between results of the H7 cELISA and an HI assay. However, the H7 cELISA reported by Sala et al. (25) also showed a nonspecific detection for the H6 antibody. The results from the present study indicate that the H7 cELISA is specific to the H7 subtype only and not to other HA subtypes (Fig. 5a). The cELISA methodology offers a promising approach for rapid, safe, and convenient serodiagnosis of H7 virus infections.

In conclusion, diagnostic applications of seven MAbs have been evaluated in this study. It has been demonstrated that MAbs produced in this study are valuable for use as diagnostic reagents to detect AI H7 virus infections.

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